Neuron 50, 381–392, May 4, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.neuron.2006.03.016

Dopaminergic Control of Corticostriatal Long-Term Synaptic Depression in Medium Spiny Neurons Is Mediated by Cholinergic Interneurons

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Summary

Long-term depression (LTD) of the synapse formed between cortical pyramidal neurons and striatal medium spiny neurons is central to many theories of motor plasticity and associative learning. The induction of LTD at this synapse is thought to depend upon D_2 dopamine receptors localized in the postsynaptic membrane. If this were true, LTD should be inducible in neurons from only one of the two projection systems of the striatum. Using transgenic mice in which neurons that contribute to these two systems are labeled, we show that this is not the case. Rather, in both cell types, the D_2 receptor dependence of LTD induction reflects the need to lower M_1 muscarinic receptor activity—a goal accomplished by D_2 receptors on cholinergic interneurons. In addition to reconciling discordant tracts of the striatal literature, these findings point to cholinergic interneurons as key mediators of dopamine-dependent striatal plasticity and learning.

Introduction

The striatum is a hub in forebrain circuits subserving both motor and cognitive functions. The principal neurons of the striatum—medium spiny neurons—integrate synaptic information from functionally diverse cortical regions, transforming it into signals that control goal-directed behaviors and habits (Graybiel et al., 1994; Yin et al., 2005). Alterations in the strength of the synaptic connections between the cortex and striatum are generally thought to underlie adaptive changes in these behaviors. The best-characterized plasticity at this synapse is long-term depression (LTD) (Centonze et al., 2001; Malenka and Bear, 2004; Nicola et al., 2000). The induction of corticostriatal LTD is dependent upon dopamine, which is released in response to reward or reward-associated stimuli (Schultz, 2005). Both molecular and pharmacological studies have provided compelling evidence that dopamine controls LTD induction by activating D_2 dopamine receptors (Calabresi et al., 1992, 1997; Kreitzer and Malenka, 2005). These receptors are positioned near glutamatergic synapses, in the membrane of medium spiny neurons (Delle Donne et al., 1997). But, this widely held model poses a conceptual problem. Medium spiny neurons are heterogeneous, forming two large, equally sized, efferent streams that differ in their axonal targets and, more importantly, in their expression of dopamine receptors (Gerfen, 1992; Surmeier et al., 1996). Neurons in only one of these groups—the striatopallidal neurons—express D_2 receptors. Is it possible that LTD is only expressed in striatopallidal medium spiny neurons and not in neighboring striatonigral neurons that rely upon D_1 dopamine receptors to signal dopamine release? Given the high percentage of neurons exhibiting LTD (Calabresi et al., 1994; Choi and Lovinger, 1997) and the centrality of LTD to theories of network plasticity, this seems highly unlikely.

Although engagement of D_2 receptors in LTD induction is clear, the location of the receptors involved is less securely fixed by experiment. Is it possible that the D_2 receptors involved are not located in medium spiny neurons, but rather in one of the interneuronal populations in the striatum? This interneuron would have to express D_2 dopamine receptors and modulate synaptic transmission in all medium spiny neurons. One interneuron fulfills these requirements: the giant striatal cholinergic interneuron. These cells express high levels of D_2 receptor protein whose activation slows autonomous pacemaking and reduces acetylcholine (ACh) release (DeBoer et al., 1996; Maurice et al., 2004; Yan et al., 1997). In turn, ACh potently modulates both major classes of medium spiny neuron (Dodt and Misgeld, 1986; Galarraga et al., 1999; Howe and Surmeier, 1995), primarily through M_1 muscarinic receptors that are strategically positioned at corticostriatal synapses (Bernard et al., 1992; Hersch et al., 1994; Yan et al., 2001). One of the major targets of M_1 receptor modulation in medium spiny neurons is the L-type Ca^{2+} channel. These channels also are positioned at the postsynaptic density of glutamatergic synapses (Day et al., 2006; Olson et al., 2005) and are necessary for LTD induction (Calabresi et al., 1994; Choi and Lovinger, 1997; Kreitzer and Malenka, 2005). M_1 receptor activation reduces the opening of these channels in response to depolarization—forging a potential antagonist link between the mechanisms underlying LTD induction and ACh.

Could the D_2 receptor dependence of LTD induction reflect the need to pause cholinergic interneuron spiking and reduce M_1 receptor tone at corticostriatal synapses? The experiments reported here provide support for this hypothesis. Using bacterial artificial chromosome (BAC) transgenic mice in which D_1 and D_2 receptor-expressing neurons are labeled with EGFP (Heintz, 2001), we show that the corticostriatal synapses in both cell types are subject to D_2 receptor-dependent LTD with similar features. More importantly, LTD at these synapses can be rescued following D_2 receptor blockade by lowering

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M_1 receptor tone. These results strongly suggest that the dopaminergic control of LTD induction at these synapses is not direct, but mediated by cholinergic interneurons.

Results

Identification of Medium Spiny Neurons in BAC Transgenic Mice

The first step toward understanding the cellular basis for the D_2 receptor dependence of LTD induction was to put in place a preparation that allowed D_1 and D_2 receptor-expressing neurons to be readily distinguished—allowing a direct test of the hypothesis that postsynaptic D_2 receptors were necessary for LTD induction. To this end, BAC transgenic mice in which cellular EGFP expression was driven by a D_1 receptor promoter construct (BAC D_1-EGFP) or by a D_2 receptor promoter construct (BAC D_2-EGFP) were obtained from GENSAT (Heintz, 2001). In striatal tissue slices from either animal, roughly half of the medium spiny neurons were labeled (Figures 1A and 1B). To verify that the labeling was specific, single-cell reverse transcription-polymerase chain reaction (scRT-PCR) profiling was performed for mRNAs associated with each cell type. As expected, EGFP-labeled medium-sized neurons from BAC D_1-EGFP mice expressed substance P (SP) and D_1 receptor mRNA, but not enkephalin (ENK) or D_2 receptor mRNAs (Figure 1C, n = 9). Conversely, labeled striatal neurons from BAC D_2-EGFP mice had detectable levels of ENK and D_2 receptor mRNA, but not SP or D_1 receptor mRNAs (Figure 1D, n = 10). Although previous work has shown that a small percentage of medium spiny neurons in young rats coexpressed D_1 and D_2 receptor mRNAs (Surmeier et al., 1996), no coexpression was detected in EGFP-labeled neurons from 3- to 4-week-old mice. Other members of the D_2 receptor class (D_3, D_4) that colocalize with D_1 receptors in medium spiny neurons were not examined, because previous work has shown that LTD induction is controlled exclusively by D_2 receptors (Calabresi et al., 1997). In both mouse lines, labeled medium-sized neurons had anatomical (Figures 1E and 1F) and electrophysiological properties that were typical of medium spiny neurons (Figures 1G and 1H; n = 5) (Kawaguchi et al., 1989).

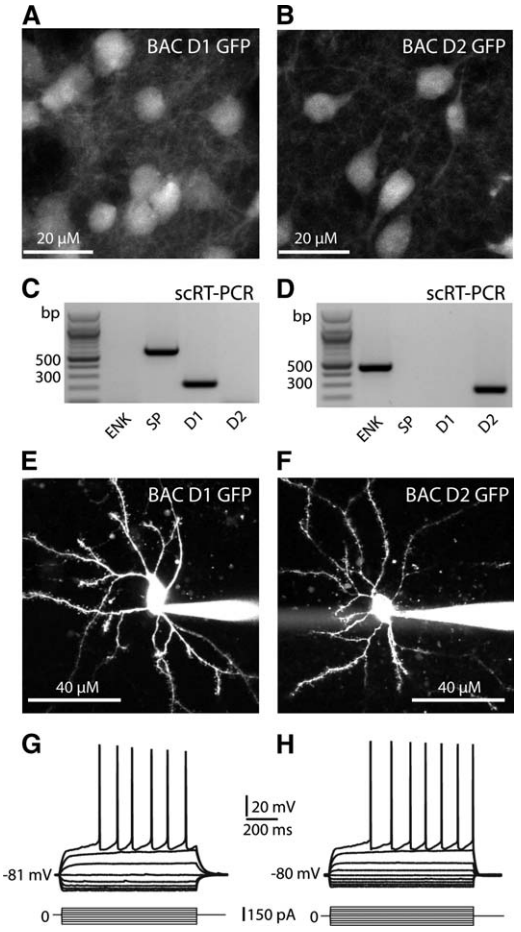

Figure 1. The Identification of Medium Spiny Neurons Expressing D_1 or D_2 Receptors in Brain Slices from BAC Transgenic Mice

(A and B) Two-photon excitation laser scanning microscopy (2PLSM) images of striatal neurons with dopamine D_1 (A) or D_2 (B) receptor labeled with EGFP in a 275 μm thick corticostriatal slice from a BAC D_1 (A) or BAC D_2 (B) mouse. 2PLSM green signals (500–550 nm) were acquired from EGFP D_1 BAC neurons using 810 nm excitation, while EGFP D_2 BAC neurons required 900 nm excitation. Scale bar, 20 μm (B).

(C and D) Single-cell RT-PCR (scRT-PCR) showing two major classes of medium spiny neurons. BAC D_1 cell coexpresses D_1 and substance P (SP) mRNA, but not D_2 or ENK mRNA. BAC D_2 cell coexpresses D_2 and enkephalin (ENK) mRNA, but not D_1 or SP mRNA.

(E and F) 2PLSM images of striatal medium spiny neurons in a corticostriatal slice were visualized with Alexa Fluor 594 (50 μM) by filling through the patch pipette. Following break-in, the dye was allowed to approach diffusion equilibrium (>15 min) prior to imaging. 2PLSM red signals (580–640 nm) were acquired using 810 nm excitation with 90 MHz pulse repetition frequency and ~250 fs pulse duration at the sample plane. Scale bar, 40 μm.

(G and H) Typical membrane responses to somatic current injection (shown below) of striatal neuron with D1 (G) or D2 (H) receptor labeled with EGFP in a corticostriatal slice from a BAC D_1 (G) or BAC D_2 (H) mouse. The medium spiny neurons showed a relatively hyperpolarized resting potential, strong inward rectification, and delayed firing. Current steps were made in 25 pA increments.

LTD Induction in Both Classes of Medium Spiny Neuron Is Sensitive to D_2 Receptor Blockade

The best-characterized form of corticostriatal LTD in medium spiny neurons is induced by pairing high-frequency stimulation (HFS) of glutamatergic afferent fibers and postsynaptic depolarization (Choi and Lovinger, 1997). This form of LTD is not dependent upon NMDA receptors. In the presence of the NMDA receptor antagonist AP-5 (50 μM) and the $GABA_A$ receptor antagonist bicuculline (10 μM), stimulation of the cortex or underlying white matter generated an EPSC in medium spiny neurons that was blocked by AMPA receptor antagonists (CNQX, 10 μM, n = *5* or NBQX, 5 μM, n = *5*) (Figure 2A), demonstrating the engagement of glutamatergic corticostriatal axons. Pairing HFS stimulation of the cerebral cortex overlying the striatum (Figure 2A) or the white matter of the corpus callosum with postsynaptic depolarization of D_2 receptor-expressing medium spiny neurons from BAC mice led to the induction of a robust LTD (Figure 2B, n = 5; see Figure S1 in the Supplemental Data available online). The LTD was similar in magnitude and kinetics to that seen in other published reports (e.g., Calabresi et al., 1992). The reduction in EPSC amplitude following LTD induction also was accompanied by an

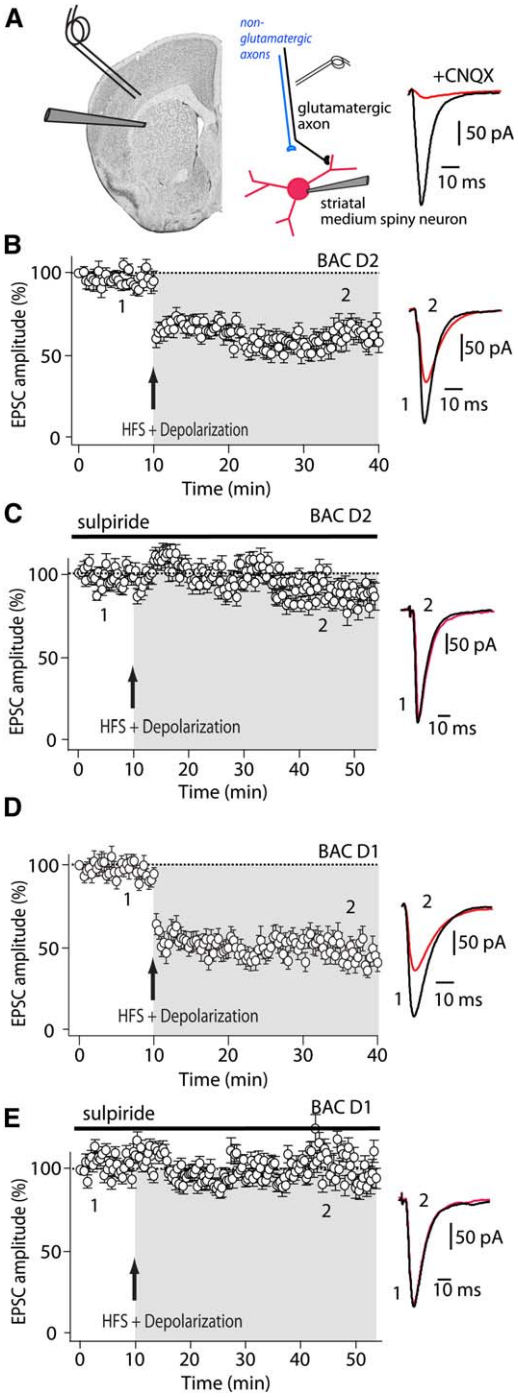

Figure 2. HFS/DP Induces Corticostriatal LTD in Both D_2 and D_1 Receptor-Expressing Medium Spiny Neurons that Is Blocked by the D_2 Receptor Antagonist Sulpiride

(A) Photomicrograph of a corticostriatal tissue slice showing the approximate areas of stimulation and recording. Cartoon representation of the cellular elements thought to be involved. Inset is an EPSC evoked by stimulation before and after AMPA receptor blockade with NBQX (10 μM). Similar results were obtained in 5 cells.

(B) LTD induced in dorsolateral striatal medium spiny neurons by HFS in combination with postsynaptic depolarization (from −70 mV to 0 mV) in labeled neurons from BAC D_2 mice (n = 5). The HFS consisted of four pulse trains (1 s at 100 Hz); the intertrain interval was 10 s. The holding potential was −70 mV. Plots of mean (±SEM) EPSC amplitude as a function of time; LTD induction was performed at the arrow. Representative current traces before and after HFS/DP from one of the cells are shown at the right.

(C) HFS/DP-induced LTD in labeled neurons from BAC D_1 mice (n = 7). Plots are as in (B).

(D) In the presence of the D_2 receptor antagonist sulpiride (10 μM), HFS/DP fails to alter mean EPSC amplitude of D_2 receptor-expressing neurons (n = 8). Plots and current traces as in (B).

(E) The D_2 receptor antagonist sulpiride (10 μM) also blocked LTD induction in D_1 receptor-expressing medium spiny neurons (n = 8). Plot and traces as in (B).

elevation in the paired-pulse ratio (PPR) (control PPR = 1.06 ± 0.07, post PPR = 1.29 ± 0.05, n = 6; $p < 0.05$ Kruskal-Wallis), suggesting a reduction in glutamate release probability (Choi and Lovinger, 1997). Moreover, the induction failed in the presence of the D_2 receptor antagonist (−) sulpiride (10 μM, Figure 2C, n = 8) (Calabresi et al., 1992). This result was obtained in two independent laboratories (data in Figure 2 is from the Surmeier lab; data in Figure S1 is from the Lovinger lab, n = 6). Because ambient extracellular levels of dopamine in slices appear to be very low (Bamford et al., 2004; Schmitz et al., 2003), it is our working assumption that the dopamine required for LTD induction is being released during HFS either by glutamate or by direct activation of dopaminergic fibers (David et al., 2005; Partridge et al., 2002).

In brain slices from BAC D_2 mice, pairing HFS and depolarization (HFS/DP) also induced a robust LTD in neighboring unlabeled (presumably D_1 receptor expressing) medium spiny neurons (Figure S1, n = 6). LTD induction in these cells also was accompanied by an elevation in PPR (Figure S1, n = 6). To provide a definitive test of whether LTD could be induced in D_1 receptor-expressing, striatonigral neurons, the BAC D_1-EGFP mice were used. In these medium spiny neurons, pairing HFS and depolarization also induced a robust LTD that was similar in kinetics and magnitude to that seen in striatopallidal neurons; LTD also was accompanied by an elevation in the PPR (control PPR = 0.97 ± 0.05, post-PPR = 1.32 ± 0.14, n = 7; $p < 0.05$ Kruskal-Wallis) (Figure 2D). As in the D_2 receptor-expressing medium spiny neurons, LTD induction in D_1 receptor-expressing medium spiny neurons was blocked by preincubation with a D_2 receptor antagonist (Figure 2E, n = 8).

LTD induced by cortical HFS/DP in medium spiny neurons is dependent upon postsynaptic generation of endocannabinoids and activation of presynaptic CB1 receptors (Gerdeman et al., 2002; Kreitzer and Malenka, 2005). In our hands, LTD induction was blocked by the CB1 receptor antagonist AM 251 (2 μM) in both D_2 (n = 7) and D_1 receptor (n = 7) expressing neurons (Figure S2).

LTD Induction Is Dependent upon Cav1.3 Ca^{2+} Channels

The ability of D_2 receptor antagonists to block LTD induction in medium spiny neurons that do not express D_2 receptors suggests that receptors on another neuron are controlling induction. One way of narrowing the range of possibilities is to identify an LTD control element that is shared by both classes of medium spiny neuron. As noted above, previous work has suggested that L-type Ca^{2+} channels are necessary for LTD induction (Calabresi et al., 1994). Recently, a subtype of L-type channel—Cav1.3 channels—was localized to spines and

postsynaptic glutamatergic synapses in both classes of medium spiny neuron (Day et al., 2006; Olson et al., 2005). Thus, these channels are appropriately positioned to regulate glutamatergic synaptic plasticity. To test for the specific involvement of Cav1.3 channels in LTD induction, two experiments were performed. First, the susceptibility of LTD to blockade of the L-type channel antagonist nimodipine was examined. Medium spiny neurons coexpress two L-type channels: one having a Cav1.2 pore-forming subunit, the other having a Cav1.3 subunit (Olson et al., 2005). These channels differ in the potency with which they are blocked by nimodipine. The IC_{50} for nimodipine block of Cav1.2 L-type channels is near 200 nM, whereas the potency of nimodipine at Cav1.3 channels is roughly 10-fold lower (Xu and Lipscombe, 2001). A concentration of nimodipine (2 μM) that should produce a near complete block of Cav1.2 channels and a 50% block of Cav1.3 channels did not block LTD induction (Figure 3A, n = 9). However, elevating the nimodipine concentration (10 μM) to produce a more complete block of Cav1.3 channels abolished LTD (Figure 3B, n = 9). Although there was no obvious heterogeneity in the response to nimodipine, BAC D_1 and D_2 medium spiny neurons were tested for their sensitivity to L-type channel blockade. In both cell types (BAC D_1, n = 3; BAC D_2, n = 4), nimodipine (10 μM) prevented LTD induction (Figure S3). Next, LTD was examined in *Cav1.3*$^{-/-}$ mice. Pairing of HFS and depolarization failed to induce any significant change in EPSC amplitude in any of these neurons (Figure 3B, n = 9). These data suggest that Cav1.3, but not Cav1.2, channels are necessary for LTD induction in both striatonigral and striatopallidal medium spiny neurons.

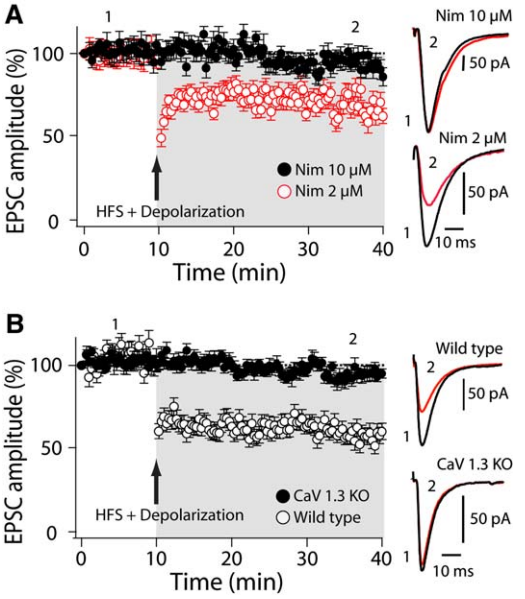

Figure 3. LTD Induction Is Dependent upon Cav1.3 Ca^{2+} Channels

(A) LTD induction in striatal medium spiny neurons was not blocked by 2 μM nimodipine, which should produce a near complete block of Cav 1.2 channels; plot of mean (±SEM) EPSC amplitude as a function of time (red circles, n = 9). Representative current traces at the right. Elevating the nimodipine concentration to 10 μM blocked LTD induction; plot of mean (±SEM) EPSC amplitude as a function of time (black circles, n = 9). Representative current traces at the right.

(B) Plot of mean EPSC amplitude (±SEM) as a function time. HFS/DP did not induce LTD in medium spiny neurons from *Cav1.3*$^{-/-}$ mice; wild-type, open circles, n = 10; *Cav1.3*$^{-/-}$ filled circles, n = 9). Representative current traces at the right.

M_1 Receptor Activity Enhances Corticostriatal Synaptic Transmission

Previous work by our group has shown that Cav1.3 channels in medium spiny neurons are potently modulated by M_1 muscarinic receptors (Howe and Surmeier, 1995; Olson et al., 2005). M_1 muscarinic receptors are robustly expressed by both D_1 receptor-expressing striatonigral and D_2 receptor-expressing striatopallidal neurons (Bernard et al., 1992; Hersch et al., 1994; Yan et al., 2001). Because cholinergic interneurons are autonomously active (Bennett and Wilson, 1999), there should be a significant level of basal ACh release and M_1 receptor activity in striatal slices. To determine whether this ambient receptor stimulation had any impact on excitatory synaptic transmission, the M_1 receptor antagonist pirenzepine (1–10 μM) was bath applied. Blockade of M_1 receptors reversibly reduced EPSC amplitude (Figure 4A, n = 7). Another way of reducing ambient M_1 receptor tone is to promote degradation of ACh by elevating the extracellular concentration of ACh esterase (AChE). Bath application of AChE (2 U/ml) also reversibly reduced EPSC amplitude (Figure 4B, n = 6).

Postsynaptic M_1 receptors might modulate synaptic transmission by reducing Cav1.3 channel opening in response to synaptic stimulation. Ca^{2+} entry through these channels is thought to control activity-dependent generation of endocannabinoids and CB1 receptor-dependent presynaptic inhibition (Gerdeman et al., 2002; Kreitzer and Malenka, 2005). Consistent with this hypothesis, pirenzepine had no effect on synaptic transmission in medium spiny neurons from Cav1.3 channel$^{-/-}$ mice (Figure S4, n = *5*). This loss was not a consequence of an adaptation to deletion of Cav1.3 subunits, as the application of nimodipine (10 μM) to wild-type medium spiny neurons either before or after pirenzepine also abolished the modulation of synaptic transmission (Figure S4, n = 5, 3). Since these channels are gated by membrane potential, postsynaptic depolarization produced by activation of ionotropic glutamate receptors should be necessary for the effect of M_1 receptor antagonism to be expressed. To test this conjecture, pirenzepine was applied and afferent stimulation stopped. As predicted, when stimulation was resumed, EPSC amplitude was at the control level, falling only with subsequent afferent activation (Figure 4C, n = 5). Presumably, afferent stimulation was necessary because it led to postsynaptic depolarization and activation of Cav1.3 channels—in spite of the somatic membrane being clamped at −70 mV. This isn't surprising, particularly if the channels are localized on spine heads where glutamatergic synaptic input is likely to produce a large transient depolarization (Carter and Sabatini, 2004). Although spine heads cannot be clamped, somatic hyperpolarization should reduce the magnitude of the depolarization achieved during synaptic stimulation, decreasing the chances of Cav1.3 channel opening. To test this conjecture, the somatic membrane potential was held at −90 mV (rather than −70 mV) during application of pirenzepine and afferent stimulation. In this situation, pirenzepine failed to alter EPSC amplitude; subsequent movement

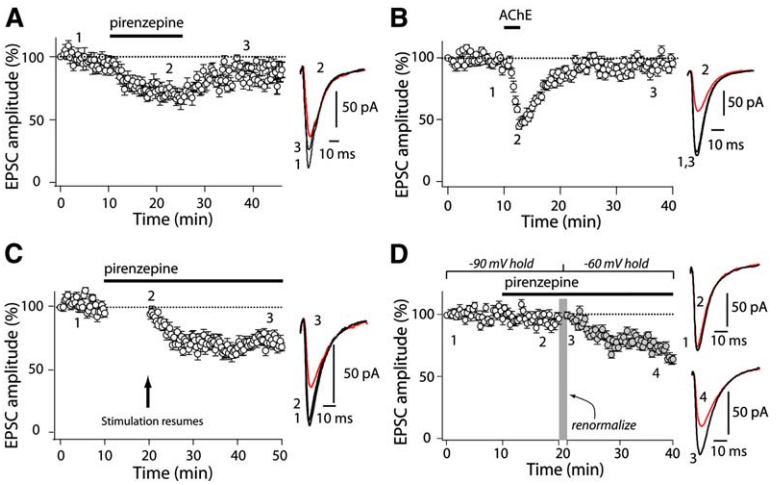

Figure 4. Lowering M1 Receptor Activity Reversibly Reduces Corticostriatal Glutamatergic Synaptic Transmission in an Activity-Dependent Manner

(A) Pirenzepine (1 μM), an M1 receptor antagonist, reversibly reduced EPSC amplitude in striatal medium spiny neurons (n = 7). Current traces in the right panel showed that pirenzepine decreased the EPSC amplitude (red) and washing out pirenzepine returned the EPSC amplitude to nearly control level.

(B) Bath application of acetylcholinesterase (AChE, 2 U/ml) reversibly reduced extracellular acetylcholine levels and EPSC amplitude in striatal medium spiny neurons (n = 6). The right panel showed current traces taken from a striatal medium spiny neuron. AChE reduced the EPSC amplitude (red) and washing out AChE brought the trace to control level.

(C) The effect of pirenzepine requires activation of the afferent terminal. Time course in the left panel showed that pirenzepine was added to the bath solution and the afferent fibers stimulation was stopped at the same time, 10 min later, when stimulation was resumed, the EPSC amplitude was at the control level and then decreased with subsequent afferent activation (n = *5*). Current traces in the right panel showed that pirenzeoine reduced the EPSC amplitude only when the afferent fibers stimulation was resumed (red). The data are shown as means ± SEM.

(D) The impact of pirenzepine on EPSC amplitude is dependent upon somatic membrane potential. As shown at the left, pirenzepine had no effect on EPSC amplitude when the somatic membrane potential was held at −90 mV. In the same cells, depolarizing the membrane potential to −60 mV allowed expression of the pirenzepine modulation (n = *5*). After changing the holding potential to −60 mV, the EPSC amplitude fell instantaneously because of the reduced driving force for the synaptic conductance; for the sake of comparison, the EPSC amplitude was renormalized using the first EPSC following the change in holding potential. This discontinuity is indicated by the gray box and the "renormalize" label. Representative current traces at the labeled time points are shown at the right. The data are shown as means ± SEM.

of the somatic membrane potential into a more depolarized, permissive range allowed expression of the modulation (Figure 4D, n = *5*). Finally, the effect of pirenzepine was associative—requiring both pre- and postsynaptic activity, as postsynaptic depolarization alone was not sufficient to enable the pirenzepine effect on synaptic transmission (data not shown, n = 3).

As mentioned above, the best characterized linkage between Cav1.3 channel opening and synaptic transmission is through endocannabinoids. The production of endocannabinoids is stimulated by elevation of cytosolic Ca^{2+}, subsequent to channel opening. As expected of such a mechanism, intracellular dialysis with the Ca^{2+} chelator BAPTA blocked the effect of pirenzepine on ESPC amplitude (Figure S4, n = *5*). To test for the involvement of CB1 receptors, the CB1 receptor antagonist AM 251 (2–5 μM) was applied prior to pirenzepine. In the presence of AM 251, lowering M_1 receptor activity with pirenzepine had no effect on synaptic transmission (Figure S5, n = 5). AM 251 also reversed the effect of AChE on synaptic transmission (Figure S5, n = 6). Taken together, these results suggest that at "rest" in the slice, ACh enhances corticostriatal synaptic transmission by activating M_1 muscarinic receptors that inhibit activity-dependent opening of Cav1.3 Ca^{2+} channels, endocannabinoid generation, and presynaptic activation of inhibitory CB1 receptors (Gerdeman et al., 2002; Kreitzer and Malenka, 2005).

LTD Induction Is Rescued by Lowering M_1 Receptor Activity following D_2 Receptor Blockade

The results presented thus far suggest that M_1 receptor antagonism facilitates signaling mechanisms known to be necessary for LTD induction. As a first step toward testing whether bath application of D_2 receptor antagonists was influencing medium spiny neurons by lowering

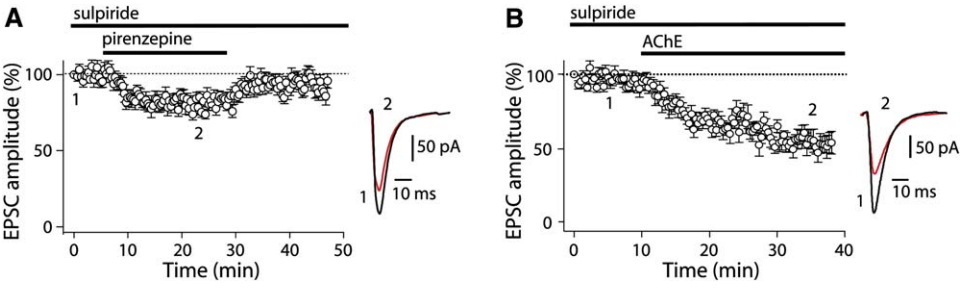

Figure 5. M_1 Receptor Control of Synaptic Transmission Is Maintained in the Presence of the D_2 Receptor Antagonist Sulpiride

(A) Preapplication of (−) sulpiride (10 μM) did not alter the pirenzepine-induced decrease in EPSC amplitude in striatal medium spiny neurons (n = 4). Plot of EPSC amplitude (±SEM) as a function of time and drug application. Representative current traces on the right.

(B) Bath application of AChE mimicked the effect of pirenzepine in the presence of sulpiride. The EPSC amplitude was decreased when AChE (2 U/ml) was added to the bath (n = 6). Plot of EPSC amplitude (±SEM) as a function of time and drug application. Representative current traces on the right.

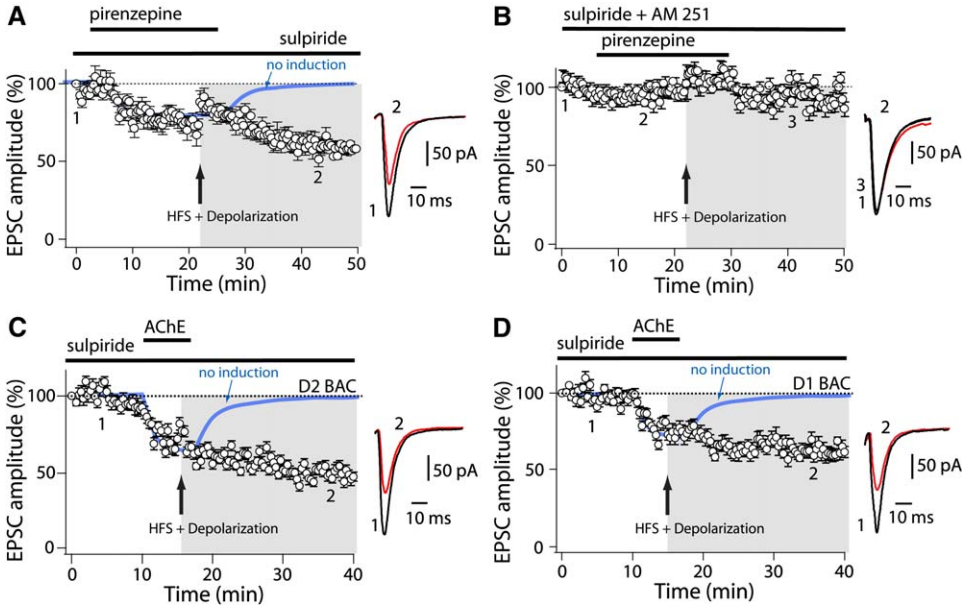

Figure 6. Lowering M_1 Receptor Activity Rescues LTD Induction in the Presence of D_2 Receptor Antagonists

(A) Plot of EPSC amplitude (±SEM) as a function of time and drug application. In the presence of (−) sulpiride and pirenzepine, HFS/DP led to LTD induction in striatal medium spiny neurons. As shown in the left panel, sulpiride (10 μM) was applied and then pirenzepine (1 μM) was added; when the pirenzepine-induced response had stabilized, the LTD induction protocol was delivered and then pirenzepine was washed out; EPSC amplitude remained at the reduced levels (n = 7). Blue line labeled "no induction" is taken from the data shown in Figure 4A, where pirenzepine was applied and then washed without HFS/DP; it was scaled to match the maximum pirenzepine modulation seen before HFS/DP and is intended only to indicate the approximate time course of EPSC recovery from the pirenzepine modulation. Current traces taken from a medium spiny neuron in the right panel showed the EPSC amplitude control (black) and after LTD induction (red).

(B) The effect of pirenzepine and LTD induction shown in (A) is dependent upon CB1 receptors, as coapplication of CB1 receptor antagonist AM 251 (2 μM) blocked the effect of pirenzepine on EPSC amplitude and LTD induction (n = 6).

(C) Plot of EPSC amplitude (±SEM) as a function of time and drug application. In the presence of sulpiride (10 μM) and AChE (2 U/ml), HFS/DP led to LTD induction in D_2 receptor-expressing striatopallidal neurons in BAC D_2 mice (n = 5). Blue line labeled "no induction" is taken from the data shown in Figure 4B, where pirenzepine was applied and then washed without HFS/DP; it was scaled to match the maximum pirenzepine modulation seen before HFS/DP and is intended only to indicate the approximate time course of EPSC recovery from the pirenzepine modulation. Panel at the right shows representative EPSC recordings control (black) and after LTD induction (red).

(D) As in (C), plot of EPSC amplitude (±SEM) as a function of time and drug application. HFS/DP-induced LTD in D_1 receptor-expressing striatonigral neurons in the presence of sulpiride and AChE (n = 4). The blue line labeled "no induction" is as in (C). Panel at the right shows representative EPSC recordings control (black) and after LTD induction (red).

ACh release, we re-examined the effects of M_1 receptor antagonism in the presence of the D_2 receptor antagonist (−) sulpiride (10 μM). As expected, sulpiride did not disrupt the modulation of synaptic transmission by pirenzepine (Figure 5A, n = 4). Bath application of AChE also mimicked the effect of pirenzepine in the presence of sulpiride, reducing EPSC amplitude by nearly 50% (Figure 5B, n = 6). Moreover, this modulation did not wane with maintained application of AChE.

If D_2 receptors were controlling LTD induction solely by lowering cholinergic tone, then reducing M_1 receptor activity should rescue LTD in the presence of D_2 receptor antagonists. To test this hypothesis, sulpiride was bath applied and then pirenzepine was added; when the pirenzepine response had stabilized, the HFS/DP protocol was delivered and then pirenzepine was washed out. Invariably, this protocol led to a lasting reduction in EPSC amplitude, indicative of LTD induction (Figure 6A, n = 7). Similar results were observed in rat striatal slices where LTD was induced by HFS/DP pairing in the presence of sulpiride and pirenzepine (Figure S6, n = 5). The properties of the LTD induced in this circumstance were similar to those seen in the absence of D_2 receptor block, arguing that this was not a novel form of LTD. Specifically, LTD induced in this circumstance (1) produced a similar reduction in EPSC amplitude, (2) was accompanied by an increase in the PPR (control PPR = 1.06 ± 0.29, post-PPR = 1.51 ± 0.26, n = 7; $p < 0.05$, Kruskal-Wallis), and (3) was blocked by CB1 receptor antagonists (Figure 6B, n = 6). The effects of HFS/DP also were examined following application of AChE. In the presence of sulpiride and AChE, HFS/DP led to robust LTD induction and an increase in the PPR. This was true in both D_2 (Figure 6C) (control PPR = 1.04 ± 0.07, post-PPR = 1.31 ± 0.18, n = 5; $p < 0.05$, Kruskal-Wallis) and D_1 receptor-expressing neurons (Figure 6D) (control PPR = 1.08 ± 0.15, post-PPR = 1.29 ± 0.12, n = 4; $p < 0.05$, Kruskal-Wallis). Thus, the principal role of dopamine and D_2 receptors in LTD induction appears to be to lower ACh release and M_1 muscarinic receptor activity in medium spiny neurons.

Discussion

D_2 Receptor Dependence of LTD Induction in Medium Spiny Neurons Is Indirect

In agreement with previous studies (e.g., Calabresi et al., 1992; Kreitzer and Malenka, 2005), our results show that

LTD induction in medium spiny neurons is dependent upon activation of D_2 dopamine receptors. This finding has long been interpreted to mean that D_2 receptors located on medium spiny neurons themselves must be activated for induction to proceed. Our data argue that this model needs to be reformulated. D_2 receptor-dependent LTD was inducible in both D_2 receptor-expressing striatopallidal neurons and D_1 receptor-expressing striatonigral neurons. In agreement with a variety of other studies (Gerfen, 1992), our single-cell RT-PCR profiling confirmed that the BAC D_1-EGFP striatonigral neurons in 3- to 4-week-old mice did not coexpress detectable levels of D_2 receptor mRNA, even though some measure of coexpression might be found at earlier developmental stages (Aizman et al., 2000). Since genetic deletion of D_2 receptors abolishes LTD (Calabresi et al., 1997), the coexpression of another member of the D_2 receptor class (e.g., D_3 receptors) in striatonigral neurons (Surmeier et al., 1996) cannot explain the sensitivity of LTD induction in these neurons to D_2-class antagonists.

One possible interpretation of these results is that there are two forms of LTD in medium spiny neurons. Certainly, the mechanisms governing LTD induction and expression vary across brain regions (Malenka and Bear, 2004). Even in medium spiny neurons, low-frequency stimulation of corticostriatal afferent fibers leads to a different form of LTD (Ronesi and Lovinger, 2005). Given the heterogeneity of the glutamatergic input to striatonigral and striatopallidal medium spiny neurons (Lei et al., 2004; Smith et al., 1998), differences in determinants of LTD induction would not be surprising. However, in the paradigm studied here, LTD induced in D_1 and D_2 receptor-expressing medium spiny neurons by pairing cortical HFS and postsynaptic depolarization had very similar determinants. In both cell types, LTD was similar in magnitude and kinetics. In both cell types, LTD was dependent upon D_2 receptors, postsynaptic L-type channels, endocannabinoids, and CB1 receptor activation. In both cell types, LTD expression was associated with an increase in the paired-pulse ratio, indicating a presynaptic locus. Clearly, the most parsimonious interpretation of these results is that the induction mechanisms are the same. The failure of previous studies to identify any heterogeneity in corticostriatal LTD or any prominent variation among medium spiny neurons in the susceptibility to HFS/DP LTD induction is consistent with this conclusion (e.g., Centonze et al., 2001; Gerdeman et al., 2002; Kreitzer and Malenka, 2005). If this conclusion is correct, induction cannot depend upon placement of D_2 receptors in the postsynaptic membrane at the corticostriatal synapse itself. Rather, the D_2 receptor dependence must be indirect, reflecting the involvement of another synaptically coupled, striatal cell type.

LTD Induction Is Dependent upon Cav1.3 Ca^{2+} Channels

A clue about the identity of this other cell came from the discovery that LTD induction depended upon a particular kind of L-type Ca^{2+} channel. Previous studies have shown that LTD was dependent upon postsynaptic L-type Ca^{2+} channels (Calabresi et al., 1994; Choi and Lovinger, 1997; Kreitzer and Malenka, 2005). Medium spiny neurons coexpress two members of this channel class, referred to as Cav1.2 and Cav1.3 on the basis of their pore-forming subunit (Olson et al., 2005; Xu and Lipscombe, 2001). The long C-terminal splice variant of the Cav1.3 subunit expressed in medium spiny neurons interacts with the scaffolding protein Shank, leading to positioning of the channel at glutamatergic synapses (Olson et al., 2005; Zhang et al., 2005). This interaction not only positions the channel near the presumptive site of LTD induction but also is responsible for maintaining a signaling interaction with M_1 muscarinic receptors, which are robustly expressed by both major classes of medium spiny neuron and are localized in spines (Bernard et al., 1992; Hersch et al., 1994; Yan et al., 2001). In the presence of Shank, M_1 receptor activation leads to a potent and selective reduction in Cav1.3 channel opening, putting these receptors in a position to negatively regulate LTD induction.

Cholinergic Interneurons—Regulators of Striatal LTD Induction?

Medium spiny neurons respond to ACh released from giant, aspiny cholinergic interneurons. These neurons are autonomous pacemakers, leading to tonic ACh release and tonic activation of striatal muscarinic receptors (Bennett and Wilson, 1999). Activation of presynaptic M_2-class receptors diminishes transmitter release in a subset of glutamatergic terminals within the striatum (Hernandez-Echeagaray et al., 1998; Hsu et al., 1995). In contrast, several studies have shown that M_1 receptor activation increases the intrinsic excitability of medium spiny neurons (Dodt and Misgeld, 1986; Galarraga et al., 1999; Howe and Surmeier, 1995). Our results add another dimension to this evolving picture by showing that M_1 receptor signaling also reversibly enhances glutamatergic synaptic function. This inference was based upon the ability of M_1 receptor antagonists and AChE to reduce excitatory EPSCs. The dependence of this modulation on Cav1.3 channels and CB1 receptors suggests that M_1 receptors promote excitatory transmission by reducing activity-dependent opening of Cav1.3 Ca^{2+} channels, which in turn diminishes endocannabinoid production and presynaptic CB1 receptor activation. Although necessary, reducing Cav1.3 channel currents was not sufficient to induce a change in synaptic transmission—coincident (or associative) pre- and postsynaptic activity was required. This observation is consistent with the absence of depolarization-induced suppression of excitatory synaptic transmission in medium spiny neurons (Gerdeman et al., 2002; Kreitzer and Malenka, 2005). Recent work suggest that the associative activity requirement stems from the need to coactivate postsynaptic metabotropic glutamate receptors to generate a sufficient level of endocannabinoid to bring about a clear presynaptic modulation (Kreitzer and Malenka, 2005).

How does this help to understand the D_2 receptor dependence of LTD? As mentioned above, D_2 receptor activation slows the autonomous spiking of interneurons and reduces ACh release. Our working hypothesis (Figure 7) is that activation of D_2 receptors is necessary for LTD only because these receptors serve to reduce ACh release and M_1 receptor tone. Lowering M_1 receptor tone promotes LTD induction by disinhibiting critical intraspine Cav1.3 Ca^{2+} channels. Direct support for this hypothesis came from the rescue of LTD induction in

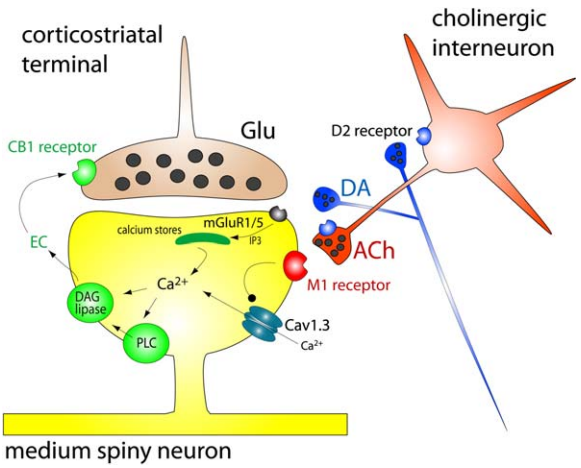

Figure 7. Model of the Cellular and Molecular Elements Controlling LTD Induction in Medium Spiny Neurons

Dopamine is hypothesized to act primarily at D_2 receptors on cholinergic interneurons, serving to lower ACh release. Lowering ACh release reduces the activity of M_1 muscarinic receptors located on spines near the site of corticostriatal glutamatergic synapses. Reducing M_1 receptor activity leads to enhanced opening of intraspine Cav1.3 Ca^{2+} channels in response to synaptic depolarization. The elevated Ca^{2+} flux results in enhanced production of endocanabinoid (EC), like 2-arachidonoylglycerol, and activation of presynaptic CB-1 receptors that reduce glutamate release.

the presence of D_2 receptor antagonists by lowering M_1 receptor tone either with an M_1 receptor antagonist or with the ACh metabolizing enzyme AChE. Again, the LTD induced in these rescue experiments relied upon the same signaling elements as LTD induced in the absence of D_2 receptor blockade (i.e., L-type channels, CB1 receptors), arguing that a novel form of plasticity was not involved.

Is there a role in LTD induction for D_2 receptors beyond reducing ACh release and M_1 receptor activity? Our data argue that postsynaptic D_2 receptors in medium spiny neurons are not necessary for induction and do not influence LTD magnitude in our recording configuration. The fact that D_2 receptors negatively couple to Cav1.3 channels in proximal dendrites, as do M_1 receptors (Hernandez-Lopez et al., 2000; Olson et al., 2005), suggests they should impede, not promote, the induction of LTD. However, in more physiological conditions, D_2 receptors might regulate intrinsic excitability in ways that would influence synaptic plasticity. It is also likely that there are other forms of LTD in medium spiny neurons that might have other determinants, including postsynaptic D_2 receptors (Malenka and Bear, 2004). D_2 receptors also appear to be present on glutamatergic terminals (Bamford et al., 2004; Cepeda et al., 2001). It seems unlikely that they play a role in the form of LTD examined here, as it is dependent upon postsynaptic mechanisms (Centonze et al., 2001; Malenka and Bear, 2004; Nicola et al., 2000) and capable of being induced in the presence of D_2 receptor antagonists. Moreover, since activation of presynaptic D_2 receptors should decrease glutamate release and postsynaptic depolarization, it is difficult to see how this would do anything but impede induction, which requires postsynaptic depolarization. Nevertheless, an involvement of these presynaptic receptors cannot be completely excluded.

Associative Learning and Striatal LTD

Our results provide a conceptual framework for reinterpreting the activity patterns of striatal cholinergic interneurons (or tonically active neurons) in associative learning paradigms. These activity patterns have been extensively characterized in primates learning an association between a reward and a light or tone (Graybiel et al., 1994; Morris et al., 2004; Schultz, 2005). As associative learning progresses, cholinergic interneurons begin to pause their activity with presentation of the conditioned light or tone. The "binding" of interneuronal activity to conditioned stimuli is thought to reflect a similar linkage between the reward-predicting value of the stimuli and the activity of dopaminergic neurons, as lesioning dopaminergic neurons abolishes the pause and learning (Aosaki et al., 1994; Schultz, 2005). This inference has been strengthened by recent work showing that the activity of dopaminergic neurons in primates perfectly mirrors the pause in interneuron activity (Morris et al., 2004). As for LTD induction, activation of D_2 dopaminergic receptors is critical to generation of the pause (Maurice et al., 2004; Watanabe and Kimura, 1998). What the pause means for striatal plasticity has been the subject of a great deal of speculation, but there has been little experimental data that bear directly on the issue. Our results suggest that one of the functions of the interneuronal pause is to transform the reward signal arising from mesencephalic dopaminergic neurons into a gating signal for LTD induction at corticostriatal synapses of both D_1 receptor-expressing striatonigral and D_2 receptor-expressing striatopallidal neurons.

Experimental Procedures

Brain Slice Preparation

Slices were obtained from 17- to 25-day-old C57BL/6 mice (Harlan), *Cav1.3*$^{-/-}$ mice (were rederived from mice obtained from Joerg Striessnig) or BAC D1/BAC D2 EGFP transgenic mice (obtained from Nathaniel Heintz), and P16–P17 rats. All animals were handled in accord with NU ACUC and NIH guidelines. Coronal slices containing the striatum were prepared at a thickness of 300–350 mm. In the Surmeier laboratory, the mice were anesthetized deeply with ketamine and xylaxine, transcardially perfused with oxygenated, ice-cold, artificial cerebral spinal fluid (ACSF), and decapitated. In the Lovinger laboratory, the mice or rats were deeply anesthetized with halothane and decapitated without perfusion. Brains were rapidly removed and sectioned in oxygenated, ice-cold, ACSF using a Leica VT1000S vibratome (Leica Microsystems). The ACSF contained the following (in mM): 124 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-(+)-glucose in the Surmeier laboratory, while in the Lovinger laboratory the modified ACSF used for sections contained (in mM) 194 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 D-glucose. The solutions were periodically checked and adjusted to ensure they stayed near 300 mOsm/l. The slices were transferred to a holding chamber where they were completely submerged in ACSF bubbled continuously with 95% O_2 and 5% CO_2 and maintained at room temperature (22°C–23°C) for at least 1 hr before recording.

Electrophysiology

Whole-cell voltage-clamp or current-clamp recordings were performed using standard techniques (Choi and Lovinger, 1997; Day et al., 2005). Individual slices were transferred to a submersion-style recording chamber and continuously superfused with ACSF at a rate of 2–3 ml/min at 31°C–33°C. Whole-cell voltage- and current-clamp recordings were performed on striatal medium spiny neurons detected in the slice with the help of an infrared-differential interference contrast (IR-DIC) video microscopy with a Olympus OLY-150 camera/controller system (Olympus, Japan). For all experiments,

10 μM (−) bicuculline methiodide or 50 μM picrotoxin was added to the superfusion medium to block $GABA_A$ receptor-mediated synaptic responses; 50 μM 2-amino-5-phosphonopentanoic acid (AP-5) was added to block NMDA receptors. Patch electrodes were made by pulling TW150F-4 (World Precisions Instruments, Sarasota, FL) or BF150-86-10 glass on a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co.) and fire polished before recording. Pipette resistance was typically 2.5–6 MW after filling with internal solution. The internal pipette solution containing the following (in mM): 120 $CsMeSO_3$, 5 NaCl, 10 TEA-Cl, 10 HEPES, 5 QX-314, 1.1 or 0.1 EGTA, 4 ATP-Mg_2, 0.3 GTP-Na_2, pH 7.2 adjusted with CsOH, 270-280 mOsm/L. For current-clamp recordings, the internal solution consisted of (in mM) 119 $KMeSO_4$, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 1 EGTA, 12 phosphocreatine, 2 ATP-Na_2, 0.7 GTP-Na_2, pH 7.2–3 with KOH, 280–300 mOsm/L. For evoked EPSC (eEPSC) recordings, medium spiny neurons located in dorsolateral striatum were voltage-clamped at −70 mV. The test stimuli were delivered at a frequency of 0.05 Hz through a concentric electrode (Frederick Haer & Co, ME) or a twisted bipolar electrode fashioned from teflon-coated tungsten wire (A-M Systems, Sequim, WA) placed in cerebral cortex or white matter near to the region of interest within the striatum. The eEPSC evoked in this situation was blocked by the AMPA receptor antagonists CNQX (10 μM, n = *5*) or NBQX (5 μM, n = *5*). Although we cannot be certain that the electrical stimulation activated only corticostriatal fibers, these fibers must be a major component of what was activated. After a stable eEPSC recording had been maintained at least for 10 min, long-term depression (LTD) was induced by high-frequency stimulation (HFS) consisted of four trains of pulses at 100 Hz, the train duration was 1 s and the intertrain interval was 10 s. During the HFS protocol, the neurons were depolarized from holding potential of −70 mV to 0 mV. The eEPSC amplitude was detected by using peak detection software provided in Clampex 8.2. The representative traces shown in the figures were the average of 10 to 15 individual sweeps in a given recording.

Two-Photon Laser Scanning Microscopy

Two-photon laser scanning microscopy (2PLSM) images of medium spiny neurons in 275 μm thick corticostriatal slices were visualized with Alexa Fluor 594 (50 μM) by filling through the patch pipette. Following break-in, the dye was loaded for at least 15 min prior to imaging. 2PLSM red signals (580–640 nm) were acquired using 810 nm excitation with 90 MHz pulse repetition frequency and ~250 fs pulse duration at the sample plane. Maximum projection images of the soma and dendritic field were acquired with a 60×/0.9 NA water-dipping lens with 0.27 μm^2 pixels and 2.6 μs pixel dwell time; ~80 images were taken using 0.7 μm focal increments. High-magnification projections of dendritic segments taken 50–100 μm from the soma were acquired with 0.17 μm^2 pixels and 10.2 μs dwell time and consisted of ~20 images taken at 0.5 μm focal steps. 2PLSM green signals (500–550 nm) were acquired from GFP^+ D1 BAC neurons using 810 nm excitation, while GFP^+ D2 BAC neurons required 900 nm excitation.

The two-photon excitation source was a Chameleon-XR tunable laser system (705 nm to 980 nm) utilizing Ti:sapphire gain medium with all-solid-state active components and a computer-optimized algorithm to ensure reproducible excitation wavelength, average power, and peak power (Coherent Laser Group). 810 nm excitation with 90 MHz pulse repetition frequency and ~250 fs pulse duration at the sample plane was used for the two-photon excitation. Laser average power attenuation was achieved with two Pockels cell electro-optic modulators (models 350-80 and 350-50, Con Optics). The two cells are aligned in series to provide enhanced modulation range for fine control of the excitation dose (0.1% steps over four decades). The laser-scanned images were acquired with a Bio-Rad Radiance MPD system (Hemel Hempstead). The fluorescence emission was collected by external or non-de-scanned photomultiplier tubes (PMTs). The green fluorescence (500–550 nm) was detected by a bialkali-cathode PMT and the red fluorescence (570–620 nm) was collected by a multi-alkali-cathode (S-20) PMT. The system digitizes the current from detected photons to 12 bits. The laser light transmitted through the sample was collected by the condenser lens and sent to another PMT to provide a bright-field transmission image in registration with the fluorescent images). The stimulation, display, and analysis software was a custom-written shareware package (WinFluor and PicViewer—John Dempster, Strathclyde University, Glasgow, Scotland, UK).

scRT-PCR

Striatal neurons from P21–P28 BAC D_1/D_2-EGFP mice were acutely isolated, harvested, and profiled for D_1 and D_2 receptor, substance P (SP), and enkephalin (ENK) using protocols similar to those previously described (Tkatch et al., 2000). The primer sequences for SP and ENK have been published (Surmeier et al., 1996; Olson et al., 2005) and have a predicted product length of 616 bp and 477 bp, respectively. D_1 mRNA (GenBank accession NM_010076) was detected with a pair of primers CTCTGCCCTACTACGAATAATG (position 1567) and CATAGTCCAATATGACCGATAAG (position 1776), which gave a PCR product of 232 bp. D_2 mRNA (GenBank accession NM_010077) was detected with a pair of primers GCTCAGGAGCTG GAAATGGAGAT (position 955) and CTTCCTGCGGCTCATCGTC TTA (position1197), which gave a PCR product of 264 bp.

Chemicals and Reagents

QX-314, (S)-(−)-sulpiride, AM 251 were obtained from Tocris. All other chemicals were from Sigma/RBI.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/50/3/443/DC1/.

Acknowledgments

This work was funded by grants from the Picower Foundation and NIH (NS 34696, MH 074866) to D.J.S., and the NIAAA Division of Intramural Clinical and Basic Research to D.M.L. We thank Dr. Rob Malenka for his helpful comments on the work. We also would like to thank Drs. Nat Heintz, Paul Greengard, and Chip Gerfen and the NINDS GENSAT program for supplying BAC transgenic mice.

Received: December 21, 2005
Revised: February 27, 2006
Accepted: April 6, 2006
Published: May 3, 2006

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